1497-Pos Board B407

Structural Differences in the Nicotinic Acetylcholine Receptor (NACHR) ion Channel Between Open and Desensitized States Revealed by Time-Resolved Photolabeling with [^{3}H]Chlorpromazine (CPZ)

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Extensive photoaffinity labeling studies and mutational analyses of *Torpedo*/muscle-type nAChR indicate only subtle differences in the structure of the ion channel between closed, open and desensitized states, with amino acids on the same face of each M2 helix lining the ion channel in each state (cytoplasmic to synaptic: M2-2, M2-6, M2-9, M2-13, M2-16/17, & M2-20). To provide further information about the differences in channel structure between the open and desensitized states, we have used time-resolved photolabeling to determine where the channel blocker [^{3}H]CPZ binds in the open channel state after 30 msec exposure to [^{3}H]CPZ and agonist, compared to its binding site(s) in nAChRs in the desensitized state exposed to [^{3}H]CPZ for 30 msec or at equilibrium. In the desensitized state, at equilibrium [^{3}H]CPZ binds to and photolabels a pocket near the cytoplasmic end of the channel (bottom, M2-2/M2-6/M2-9) and a pocket near the extracellular end of the channel (top, M2-16/17/M2-20), while in the closed, resting state it photolabels only the site at the bottom of the channel [Chiara,DC, *et.al.Biochemistry* 2009 **48**:10066-77]. For nAChRs pre-equilibrated with agonist (desensitized state), after 30 msec [^{3}H]CPZ photolabels only the site at the top of the channel (M2-16/17/20), while for nAChRs in the open state (30 msec agonist and CPZ), the pockets at both the bottom (M2-2/M2-6/M2-9) and top (M2-16/17/M2-20) were photolabeled. From these results we conclude that: 1) a structural alteration at the top of the ion channel occurs between closed and open states, whereas this region is similar in structure in the open and desensitized states; and 2) in the equilibrium desensitized state, there is an access barrier for CPZ at or near M2-13 (Supported by GM58448).

1498-Pos Board B408

Assay Development and Validation for the Muscle Nicotinic Receptor Using the Qpatch HTX™ Automated Patch Clamp System

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QPatch HTX is an automated patch clamp system with greatly increased throughput over conventional patch clamp methods. In this study, we evaluated the suitability of this system for the characterization of the fast desensitizing ligand-gated ion channel coupled with alpha1 nicotinic acetylcholine receptors (α1nAChR). HEK293 cells stably expressing human α1nAChR were obtained from Millipore. All experiments were carried out with the QPatch HTX, which performs 48 parallel and independent patch-clamp recordings on a disposable QPlate X with 10 holes per measurement site. Brief applications of acetylcholine (ACh) produced fast inward currents characteristic of the α1nAChR recorded by conventional patch clamp in heterologous expression systems and the native channel. The EC_{50} values for ACh ranged from 3 to 10 μM (n=10). Nicotine and epibatidine also elicited inward currents with EC_{50} values of 110 μM and 4 μM, respectively (n=6 & 12). The ACh-induced current was resistant to bath applications of atropine up to 1 μM and muscarine did not elicit a response, showing that the ACh-induced inward current is mediated by nAChR. Non expressing HEK293 cells did not respond to ACh. The ACh-mediated currents were blocked by α-bungarotoxin and pancuronium in a dose dependent manner (IC_{50}= 80 and 0.8 nM, respectively).

In conclusion, this study demonstrated that fast desensitizing ligand-gated ion channel can be efficiently targeted with QPatch HTX system for characterization of physiological and pharmacological properties.

1499-Pos Board B409

NMR Study of General Anesthetic Interactions with the Neuronal N-Acetylcholine Receptor Transmembrane Domain

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General anesthetics have been shown to inhibit neuronal nicotinic acetylcholine receptors (nAChRs) channel function but the mechanisms of this action are not very clear. Here we present the study of the interactions of the anesthetics halothane, isoflurane, and ketamine with the transmembrane domains of the human nAChRs $(\alpha_4)_2(\beta_2)_3$ and $(\alpha_4)_3(\beta_2)_2$ using high-resolution solution NMR. The isolated transmembrane domains of α_4 and β_2 subunits, with the extracellular and intracellular domains removed by mutagenesis, were expressed in E. coli, purified into detergent micelles and mixed in the indicated ratio. Residues affected by anesthetics were determined on the basis of their chemical shift changes in ^{15}N-^{1}H HSQC spectra upon titration by anesthetics. In addition to intrasubunit binding sites identified recently, anesthetic interaction sites at the subunit interfaces were also detected. The main site was located near the extracellular interface involving the beginning of TM1 of one of the subunits and the loop between TM2 and TM3 (or residues near the loop) of the other subunit. Moreover, two leucines in the middle of TM2 (one of which facing the pore is the putative hydrophobic gate of the channel) were also significantly affected by anesthetics. Both interaction sites were common for halothane and isoflurane. The intravenous anesthetic ketamine affected a number of residues in TM4, suggesting that ketamine could bind at the protein-detergent interface. The ketamine binding site at the extracellular interface appeared to be similar to the halothane or isoflurane one. Anesthetics also affected NMR peak intensity and line width of specific residues and in some cases resulted in peak splitting due to conformational exchange, indicating modulations of protein dynamics on the microsecond-to-millisecond timescale, which directly related to protein function. This work was supported by NIH grants: R01GM066358, R01GM056257, and R37GM049202.

1500-Pos Board B410

Rigid Motion Near the $GABA_A$ Receptor α_1 Subunit First Transmembrane Segment Revealed by pCMBS$^-$ Reactivity in Cysteine-Substituted Mutants During Channel Activation

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The gamma-aminobutyric receptor ($GABA_AR$) belongs to the pentameric ligand-gated ion channels (pLGIC) family and mediates inhibitory synaptic transmission in the brain. GABA binding in the extracellular domain triggers a conformational change that results channel gating. The molecular details of the events linking the agonist binding site and the gate, distant by ~60 Å, remain uncertain. AChBP crystal structures obtained in the absence and presence of ligand showed that the C-loop undergoes large movement "capping" the agonist in its binding site. The C-loop is directly connected to the first transmembrane segment (M1) of the receptor by the extracellular β10 β-strand. We hypothesize that C-loop movement during gating could be transmitted to M1. We sought to investigate the mobility of the M1 segment of the $GABA_AR$ α_1 subunit during gating using SCAM. We measured the rates of reaction of the cysteine-specific reagent pCMBS$^-$ in various Cys-substituted receptors in the closed and the activated states. The effects of pCMBS$^-$ were followed using two electrode voltage clamp of Xenopus oocytes injected with mRNA coding for the Cys-substituted $GABA_AR$. pCMBS$^-$ reaction rate is proportional to the solvent accessibility of the substituted residue. The results indicate that the first part of M1 is completely solvent accessible. The most reactive residues are located on one face of the α-helix and the rate of reaction increases significantly with activation. The distribution of rapidly reacting residues does not differ significantly between the closed state and the activated state. This face is directed toward M3 in the adjacent β_2 subunit, forming the general anesthetic binding site. We suggest that βM3 moves past αM1 pushing lipid away from the fast reacting face of αM1 thereby increasing the pCMBS reaction rate.

1501-Pos Board B411

On the Mechanism Underlying the Irreversible Desensitization of Human Ganglionic Nicotinic Receptors

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alpha3-containing nicotinic acetylcholine receptors (AChRs) are expressed in autonomic ganglia and are thought to mediate fast synaptic transmission. These receptors have largely eluded rigorous kinetic characterization due to a combination of poor expression in heterologous systems and the total loss of receptor responsiveness during the course of excised-patch electrophysiology experiments. Recently, it has been demonstrated that a cysteine-to-alanine mutation at the −4' position of the cytosolic M1-M2 linker can prevent this rundown in whole-cell patches of rodent superior cervical ganglion neurons (Campanucci et al., 2010). This work also suggested that this rundown, which plays a pivotal role in diabetic neuropathy, is due to oxidation of these cysteine residues. Here, we heterologously expressed human alpha3-subunit-containing AChRs in HEK-293 cells and found that the −4' cysteine mutation cannot prevent the irreversible rundown of currents observed in outside-out patches of membrane. Furthermore, we found that the irreversible loss of receptor responsiveness does not occur in the whole-cell or cell-attached configurations, implying that patch excision leads to this phenomenon. We also investigated the effects of the reciprocal −4' cysteine mutation in all five subunits of the human muscle AChRs, which do not natively have cysteines at this position. We found that introduction of the cysteines does not lead to an irreversible rundown, although it does slow down the rate of recovery from desensitization. Taken together, our results thus far suggest that the mechanism underlying the rundown of currents mediated by alpha3-containing AChRs in outside-out patches is fundamentally different from that mediated by cysteine oxidation in whole-cell recordings.